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*Original Article*

# Association of immune-related gene DNA methylation patterns with tumor progression in colorectal cancer: a clinical translational study

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**Abstract:** Objective: To evaluate the prognostic value and clinical use of plasma-derived immune gene DNA methylation patterns in colorectal cancer (CRC). Methods: A total of 300 patients with pathologically confirmed CRC (2021-2023) and 100 healthy controls were retrospectively enrolled. Methylation levels of programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), interferon regulatory factor 1 (IRF1), and forkhead box protein P3 (FOXP3) were measured using methylation-specific quantitative polymerase chain reaction (MSP). An integrated methylation risk score (IMRS) was developed using a logistic regression model. The associations between IMRS and clinicopathologic characteristics were analyzed. All patients were followed up for 24 months postoperatively. The prognostic performances of IMRS and carcinoembryonic antigen (CEA) for recurrence-free survival (RFS) were evaluated and compared using Kaplan-Meier and Cox regression analyses. Results: CRC patients exhibited a markedly elevated IMRS compared to controls ($P < 0.001$). Elevated IMRS was significantly correlated with advanced Tumor-Node-Metastasis (TNM) stage ($P = 0.002$), lymph node metastasis ($P = 0.005$), and distant organ metastasis ($P = 0.001$). Survival analysis revealed a 2-year RFS rate of 54.0% in the high-IMRS patients, significantly lower than 88.0% in the low-IMRS cohort (Log-rank $P < 0.001$). After adjustment for TNM stage, CEA, and other confounders, multivariate Cox regression analysis identified high IMRS as an independent predictor of poorer RFS (hazard ratio (HR) = 2.81, 95% confidence interval (CI): 1.88-4.20). The predictive value of IMRS (C-index = 0.79) outperformed TNM stage, preoperative CEA, and individual gene methylation markers. Conclusions: The IMRS, derived from circulating immune gene methylation, independently predicts aggressive clinicopathological features and unfavorable prognosis in CRC, highlighting its potential as a noninvasive biomarker for postoperative risk stratification.

**Keywords:** Colorectal cancer, DNA methylation, immune-related genes, clinical translation, tumor progression, prognostic markers

## Introduction

Colorectal cancer (CRC) is a major contributor to global cancer mortality [1]. Although surgical resection combined with adjuvant therapy has significantly improved patient outcomes, postoperative recurrence remains a major clinical challenge, significantly compromising long-term survival [2, 3]. Currently, postoperative risk stratification relies primarily on traditional indicators, including pathologic Tumor-Node-Metastasis (TNM) stage and serum carcinoembryonic antigen (CEA) [4]. However, the predictive performance of these approaches remains suboptimal. Patients within the same TNM stage often exhibit heterogeneous outcomes, and CEA demonstrates suboptimal sensitivity and specificity [5]. Consequently, there is an urgent need to identify novel prognostic biomarkers that can more accurately and noninvasively reflect tumor biology and facilitate individualized treatment.

Liquid biopsy techniques, especially those based on circulating cell-free deoxyribonucleic acid (cfDNA) in peripheral blood, have emerged

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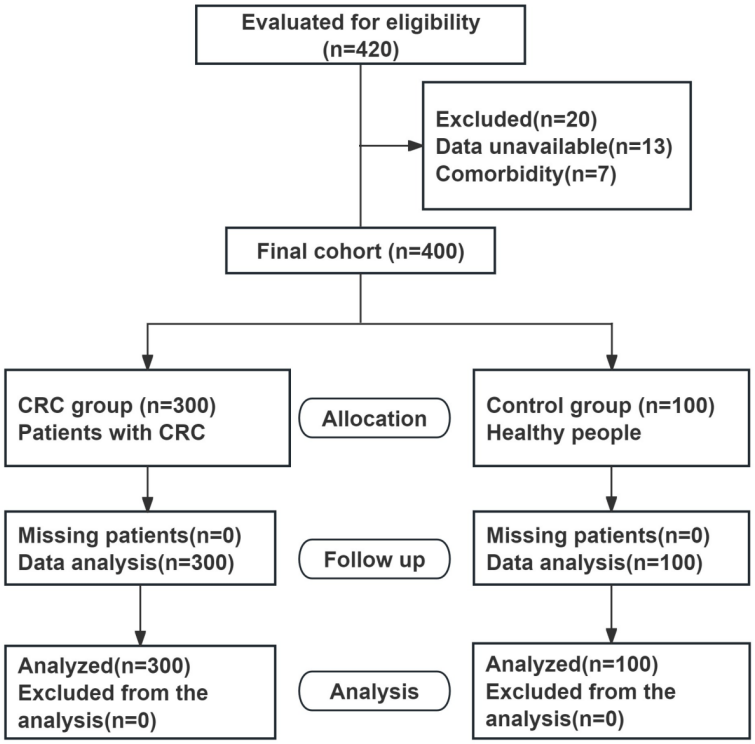


**Figure 1.** Study flow chart.

as revolutionary tools for noninvasive dynamic tumor monitoring [6]. Among them, DNA methylation, as a stable epigenetic modification, plays a key role in the occurrence and progression of tumors, and can be sensitively detected in circulating cfDNA [7]. Prior research indicated that the methylation status of specific genes correlated with CRC progression [8]. However, most studies focused on single or limited numbers of genetic markers, and their predictive efficacy and stability were often insufficient to comprehensively capture the high heterogeneity of tumors [9].

Tumor progression is closely related to dynamic changes in the immune microenvironment [10, 11]. Epigenetic regulation of key immune-related genes, such as programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), particularly through DNA methylation, has been recognized as a critical mechanism modulating tumor immune responses [12]. Therefore, systematic evaluation of functionally relevant immune gene methylation profiles and their integration into a comprehensive scoring system may provide a more comprehensive assessment of tumor immune evasion and aggressiveness, going, potentially offering beyond traditional single markers [13]. However, the clinical translational utility of multi-gene cfDNA methylation-based composite scores for CRC prognosis needs to be fully verified in well-designed clinical cohorts.

In this retrospective cohort study, we aimed to evaluate the relationship between an integrated methylation risk score (IMRS), derived from four immune-related genes (PD-L1, CTLA-4, interferon regulatory factor 1 (IRF1), forkhead box protein P3 (FOXP3)), and clinicopathologic profiles as well as recurrence-free survival (RFS) of CRC patients. We hypothesized that IMRS could effectively identify aggressive CRC phenotypes and serve as an independent predictor of postoperative RFS. This study further seeks to evaluate the feasibility of IMRS as a risk stratification tool for postoperative recurrence, thereby providing a novel basis for precision prognostic assessment of CRC.

## Materials and methods

### *Study design*

This retrospective cohort study was conducted to evaluate the association between DNA methylation of immune-related genes and the prognosis and disease progression of CRC. The study protocol was approved by the Ethics Committee of The First Hospital of Shanxi Medical University (Approval No. NOKYYJ-2025-256). Given the retrospective nature of the study, the requirement for informed consent was waived. All procedures were performed in accordance with the Declaration of Helsinki and relevant international guidelines for medical research. Clinical data were retrospectively collected from patients with pathologically confirmed CRC and contemporaneous healthy controls at our hospital between January 2021 and December 2023. The overall study workflow is summarized in **Figure 1**.

*Inclusion criteria*

CRC group: ① Histopathologically diagnosed colorectal cancer according to the Chinese Guidelines for Diagnosis and Treatment of Colorectal Cancer (2018 edition) [14]; ② Age between 18 and 75 years; ③ No prior anticancer treatment, including radiotherapy, chemotherapy, or immunotherapy, at the time of diagnosis; ④ Complete clinical data available.

Control group: ① Healthy individuals undergoing routine physical examinations during the same period, matched to the CRC group by age and sex at a ratio of 1:3; ② No abnormalities in physical examination, blood routine examination, tumor markers (including CEA), or abdominal imaging; ③ No history of malignant tumors or autoimmune diseases.

*Exclusion criteria*

① Severe organ dysfunction (e.g., heart, liver, kidney, or lung); ② History of autoimmune or chronic inflammatory bowel diseases (e.g., ulcerative colitis, Crohn's disease); ③ Recent steroid therapy and immunosuppressive therapy; ④ Previous history of other malignant tumors or family history of cancer (first-degree relatives diagnosed with malignant tumors); ⑤ Lack of clinical data to complete the statistical analysis; ⑥ Pregnant or lactating women.

*Sample size calculation*

The sample size was calculated based on differences in immune gene methylation rates reported in preliminary experiments and previous studies [15]. Assuming an overall methylation positivity rate of approximately 60% in the CRC group and 10% in healthy controls, with a statistical power (1-β) of 90% and a two-sided significance level (α) of 0.05, the minimum required sample size was calculated to be 88 subjects per group. This calculation used the formula for comparing two independent proportions.

To meet the requirements of statistical power for multivariate survival analysis, subgroup analysis, and possible loss to follow-up, a total of 300 CRC patients and 100 healthy controls were ultimately included. The sample size exceeded the minimum requirement and was considered sufficient to ensure the robustness of statistical analyses in this retrospective exploratory study.

*Study methods*

All biological samples and clinical data were collected and analyzed retrospectively. The detailed experimental procedures were as follows:

Sample collection: Peripheral blood samples were obtained from all participants at enrollment. 5 mL of fasting peripheral venous blood was collected in the morning using ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes. Within 2 hours of collection, samples were centrifuged at 2000×g for 10 minutes at 4°C. The plasma fraction was carefully separated, aliquoted into sterile nuclease-free cryovials, and stored at -80°C until further analysis.

DNA extraction and bisulfite conversion: cfDNA was extracted from plasma samples using a commercial Kit (QIAamp Circulating Nucleic Acid Kit, Qiagen, Hilden, Germany, Cat. No. 55114) according to the manufacturer's instructions. The extracted cfDNA was then subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA, Cat. No. D5005), which converts unmethylated cytosines into uracil while preserving methylated cytosines.

Methylation-specific polymerase chain reaction (PCR) (MSP): Methylation levels in the promoter or CpG island regions of PD-L1, CTLA-4, IRF1, and FOXP3 were detected using SYBR Green-based methylation-specific real-time PCR. The methylation-specific primers for each gene are listed in Supplementary Table 1. Beta-actin (ACTB)was used as the reference control to normalize DNA input. Each 20 μL reaction contained 10 μL of 2× SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, USA, Cat. No. A25741), 500 nM each primer, and 20 ng bisulfite-converted DNA. Amplification was performed on a real-time PCR system (LightCycler 480, Roche Diagnostics, Germany) under the following conditions: 50°C for 2 min (UDG incubation), 95°C for 2 min (Taq activation), followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Melting curve analysis was performed to confirm amplification specificity. The fold change in meth-

ylation level was calculated using the ΔCt method, where ΔCt = Ct(target gene) - Ct(ACTB), and fold change = $2^{-\Delta\Delta Ct}$, with ΔΔCt = ΔCt(sample) - ΔCt(mean of healthy controls). Results were expressed as fold change relative to healthy controls, with the mean of the healthy control group defined as 1. Each sample was analyzed in triplicate to ensure technical repeatability.

*Data collection*

Baseline characteristics, including demographic variables (age, sex, body mass index [BMI], smoking history, and alcohol consumption), comorbidities (hypertension, type 2 diabetes mellitus [T2DM]), and preoperative laboratory parameters (CEA, carbohydrate antigen 19-9 (CA19-9), neutrophil to lymphocyte ratio (NLR), albumin and hemoglobin), of all subjects were collected from the hospital's electronic medical record system.

For the CRC group, additional clinicopathologic characteristics were collected, including pathologic type, TNM stage according to the American Joint Committee on Cancer (AJCC) 8th edition [16], lymph node involvement, distant metastasis, tumor differentiation, tumor size, and tumor location. Preoperative serum levels of CEA and CA19-9 were measured using electrochemiluminescence immunoassays on the Cobas E 801 immunoassay analyzer (Roche Diagnostics, Mannheim, Germany). The CEA assay was performed using the Elecsys CEA reagent kit (Roche Diagnostics, Cat. No. 09015736190 for cobas e 801), and the CA19-9 assay using the Elecsys CA 19-9 reagent kit (Roche Diagnostics, Cat. No. 08244787190 for cobas e 801). Both assays were conducted according to the manufacturer's instructions, with reference ranges of 0-5 ng/mL for CEA and 0-37 U/mL for CA19-9. Complete blood counts, including neutrophil and lymphocyte counts, were determined using an automated hematology analyzer (Mindray BC-6000 series, Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China). The NLR was subsequently calculated as the absolute neutrophil count divided by the absolute lymphocyte count.

*Observational outcomes*

*Primary outcomes:* (1) IMRS: Based on the methylation level data of four genes (PD-L1, CTLA-4, IRF1, FOXP3), a multivariate logistic regression model was constructed with patient status (CRC vs. healthy control) as the dependent variable. IMRS was calculated, in which the regression coefficient of each gene served as its corresponding weight. The IMRS = Σ (methylation level of gene i × its corresponding regression coefficient i) [17]. (2) RFS: RFS was defined as the time from radical surgery to the first documented recurrence, metastasis, or death [18]. Patients without an event at the end of follow-up were censored at their last follow-up date.

*Secondary outcomes:* (1) Individual gene methylation level: The relative methylation rate of each immune-related gene was quantified by qMSP. (2) Clinicopathological characteristics: Age, sex, tumor location, histologic differentiation grade, T/N/M stage, overall TNM stage, and preoperative CEA level (< 5 ng/mL). (3) Correlation between IMRS and clinicopathologic features: The correlation between IMRS and the above clinicopathologic variables were analyzed. (4) Comparison of predictive performance: The predictive performances of IMRS, each single gene methylation marker, and preoperative CEA level for RFS in CRC patients were evaluated and compared.

*Statistical analysis*

All statistical analyses were performed using SPSS (version 26.0) and R (version 4.0.3). All tests were two-sided, and a $p$ value < 0.05 was considered significant.

Continuous variables with a normal distribution were expressed as mean ± standard deviation and compared using independent t-tests. Continuous variables not conforming to normal distribution were expressed as median (interquartile range) and analyzed using the Mann-Whitney U test. Categorical variables were reported as frequencies (percentages) and compared using the chi-square test or Fisher's exact test, as appropriate. The IMRS was constructed using a multivariable logistic regression model, with patient status (CRC = 1, control = 0) as the dependent variable and the methylation levels of the four genes as independent variables. Model parameters were estimated using maximum likelihood estimation. Model calibration was assessed using the Hosmer-Lemeshow goodness-of-fit test, with a

*p*-value > 0.05 indicating adequate calibration. Discriminative ability for distinguishing CRC patients from healthy controls was evaluated using the area under the receiver operating characteristic curve (AUC).

Based on the median IMRS, CRC patients were stratified into high- and low-IMRS groups. Associations between IMRS groups and clinicopathologic characteristics were assessed using the chi-square test. RFS was estimated using the Kaplan-Meier method, with group comparisons made using the Log-rank test. Prognostic factors for RFS were evaluated using univariate and multivariate Cox proportional hazards regression models. Variables with P < 0.10 in univariate analysis were included into the multivariate model. Hazard ratios (HR) and corresponding 95% confidence intervals (CI) were calculated. The C-index (concordance index) was used to assess and compare the discriminative performance of each indicator, with higher values indicating better predictive accuracy.

## Results

*Baseline characteristics*

A total of 300 CRC patients and 100 healthy controls were included in this study. The CRC and control groups were comparable in age, sex, BMI, lifestyle factors, and comorbidities (all *P* > 0.05); however, CRC patients had significantly higher CEA, CA19-9, NLR, and lower albumin and hemoglobin (all *P* < 0.001; **Table 1**), confirming balanced and comparable baseline demographics, comorbidities, and preoperative laboratory parameters. Among CRC patients, clinicopathologic analysis revealed that 55.0% (165/300) were classified as stage III-IV, in which 61.7% of the tumors were distributed in the colon and 66.0% exhibited deep tumor invasion (T3-T4 stage). Elevated preoperative CEA (> 5 ng/mL) were observed in 39.3% of patients.

Notably, the proportion of elevated CEA was significantly higher in stage IV patients (82.5%, 47/57) compared to those in stage I-III (29.2%, 71/243). Similarly, poorly differentiated tumors were more frequent in stage IV patients (43.9%, 25/57) than in stage I-III patients (30.5%, 74/243).

*Methylation levels of immune-related genes*

The cfDNA methylation levels of the PD-L1, CTLA-4, IRF1, and FOXP3 were significantly higher in CRC patients compared to controls (all *P* < 0.001; **Table 2**).

*Integrated immune-related gene methylation score (IMRS)*

Based on the logistic regression model, the IMRS was calculated using the following formula: IMRS = (0.124 × PD-L1 methylation) + (0.096 × CTLA-4 methylation) + (0.088 × IRF1 methylation) + (0.085 × FOXP3 methylation). All four regression coefficients were statistically significant (all *P* < 0.001). The logistic regression model demonstrated good discriminative ability, with an AUC of 0.86 (95% CI: 0.82-0.90) for distinguishing CRC patients from healthy controls. The Hosmer-Lemeshow test yielded a *p*-value of 0.324, indicating satisfactory calibration.

As shown in **Table 3**, the median IMRS was significantly higher in CRC patients (2.15 [1.28, 3.42]) than in healthy controls (1.00 [0.72, 1.38]) (*P* < 0.001; **Table 3**). Using the median value (2.15) as the cutoff, CRC patients were stratified into high- (n = 150) and low-IMRS (n = 150) subgroups.

*Correlation between IMRS and clinicopathologic features*

Correlations between IMRS categories and clinicopathologic features are summarized in **Table 4**. No significant differences were observed between the high- and low-IMRS groups regarding age, sex, tumor location, or differentiation grade (all *P* > 0.05). However, patients with high IMRS exhibited more aggressive features, including advanced TNM stage (III-IV: 66.0% vs. 44.0%, *P* = 0.002), deeper tumor invasion (T3-T4: 72.7% vs. 59.3%, *P* = 0.015), increased lymph node metastasis (56.0% vs. 40.7%, *P* = 0.005), higher incidence of distant metastasis (26.0% vs. 12.0%, *P* = 0.001), and elevated preoperative CEA levels (> 5 ng/mL: 46.7% vs. 32.0%, *P* = 0.008).

*Association of IMRS with RFS in CRC patients*

Kaplan-Meier analysis was performed to evaluate the prognostic value of IMRS for RFS (**Figure**

**Table 1.** Comparison of demographic and clinical characteristics between the two groups

| Indicator | CRC Group (n = 300) | Control Group (n = 100) | Statistical | *p*-value |
|---|---|---|---|---|
| Demographics | | | | |
| Age (years), mean ± SD | 62.5 ± 10.8 | 60.1 ± 9.5 | t = 1.89 | 0.06 |
| Male sex, n (%) | 172 (57.3) | 55 (55.0) | $\chi^2$ = 0.21 | 0.646 |
| Body Mass Index (kg/m$^2$), mean ± SD | 24.2 ± 3.5 | 23.8 ± 3.1 | t = 1.05 | 0.295 |
| Current or past smoker | 85 (28.3) | 23 (23.0) | $\chi^2$ = 1.10 | 0.294 |
| Current or past alcohol consumer | 79 (26.3) | 22 (22.0) | $\chi^2$ = 0.75 | 0.387 |
| Comorbidities, n (%) | | | | |
| Hypertension | 102 (34.0) | 28 (28.0) | $\chi^2$ = 1.33 | 0.249 |
| Type 2 Diabetes Mellitus | 48 (16.0) | 11 (11.0) | $\chi^2$ = 1.60 | 0.206 |
| Preoperative Laboratory Measures | | | | |
| Elevated CEA (> 5 ng/mL), n (%) | 118 (39.3) | 5 (5.0) | $\chi^2$ = 45.21 | < 0.001 |
| Elevated CA19-9 (> 37 U/mL), n (%) | 67 (22.3) | 3 (3.0) | $\chi^2$ = 19.87 | < 0.001 |
| NLR, median (IQR) | 2.5 (1.8, 3.6) | 1.7 (1.4, 2.1) | Z = 8.92 | < 0.001 |
| Albumin (g/L), mean ± SD | 38.5 ± 4.2 | 42.1 ± 3.5 | t = 8.01 | < 0.001 |
| Anemia (< 120 g/L), n (%) | 89 (29.7) | 10 (10.0) | $\chi^2$ = 15.67 | < 0.001 |
| Tumor Characteristics (CRC Group Only) | | | | |
| Location, n (%) | | | - | - |
| Colon | 185 (61.7) | - | - | - |
| Rectum | 115 (38.3) | - | - | - |
| Differentiation Grade, n (%) | | | - | - |
| Well/Moderate | 201 (67.0) | - | - | - |
| Poor/Undifferentiated | 99 (33.0) | - | - | - |
| TNM Stage, n (%) | | | - | - |
| Stage I | 68 (22.7) | - | - | - |
| Stage II | 67 (22.3) | - | - | - |
| Stage III | 108 (36.0) | - | - | - |
| Stage IV | 57 (19.0) | - | - | - |
| T Stage, n (%) | | | | |
| T1-T2 | 102 (34.0) | - | - | - |
| T3-T4 | 198 (66.0) | - | - | - |
| Lymph Node Metastasis (N), n (%) | | - | - | - |
| N0 | 155 (51.7) | - | - | - |
| N1-N2 | 145 (48.3) | - | - | - |
| Distant Metastasis (M), n (%) | | - | - | - |
| M0 | 243 (81.0) | | | |
| M1 | 57 (19.0) | | | |
| Preoperative CEA Level, n (%) | | - | - | - |
| ≤ 5 ng/mL | 182 (60.7) | - | - | - |
| > 5 ng/mL | 118 (39.3) | - | - | - |

Note: CRC: colorectal cancer; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; NLR: neutrophil-to-lymphocyte ratio; IQR: interquartile range; SD: standard deviation; TNM: Tumor-Node-Metastasis. Continuous data (mean ± SD) were compared using independent t-tests; categorical data [n (%)] by Chi-square test. The Mann-Whitney U test was applied to NLR (non-normal distribution).

**2** and **Table 5**). Survival analysis showed that during the 24 months of follow-up, 69 cases in the high IMRS group (150 cases) and 18 cases in the low IMRS group (150 cases) experienced recurrence ($P < 0.05$). The 2-year RFS rate was 54.0% in the high-IMRS group, compared to 88.0% in the low-IMRS group. Kaplan-Meier survival curves showed significant separation

**Table 2.** Comparison of target gene methylation levels between CRC and control groups

| Gene | CRC Group, Median (IQR) | Control Group, Median (IQR) | Z score | *p*-value |
|---|---|---|---|---|
| PD-L1 | 3.82 (1.65, 7.21) | 1.00 (0.62, 1.42) | -12.45 | < 0.001 |
| CTLA-4 | 4.65 (2.18, 9.33) | 1.00 (0.65, 1.38) | -13.21 | < 0.001 |
| IRF1 | 3.58 (1.42, 6.75) | 1.00 (0.60, 1.41) | -11.88 | < 0.001 |
| FOXP3 | 4.12 (1.89, 8.06) | 1.00 (0.63, 1.40) | -12.67 | < 0.001 |

Note: CRC, colorectal cancer; IQR, interquartile range; PD-L1, programmed death-ligand 1; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IRF1, interferon regulatory factor 1; FOXP3, forkhead box protein P3. Data are expressed as median (IQR). Intergroup comparisons utilized the Mann-Whitney U test.

**Table 3.** Comparison of IMRS between study groups

| Group | n | IMRS, Median (Q1, Q3) | Z score | *p*-value |
|---|---|---|---|---|
| CRC Group | 300 | 2.15 (1.28, 3.42) | -15.34 | < 0.001 |
| Control Group | 100 | 1.00 (0.72, 1.38) | - | - |

Note: IMRS: Immune-related gene methylation integrated score; Data are presented as median (first quartile, third quartile); Between-group differences were assessed with the Mann-Whitney U test. The Z score and *p*-value correspond to the overall comparison result.

between the two groups ($P < 0.001$), indicating that higher IMRS was significantly associated with poorer RFS.

*Univariate and multivariate Cox regression analysis*

To assess the independent prognostic value of IMRS for RFS, Cox proportional hazards regression analysis was conducted (**Table 6**). Univariate analysis identified advanced TNM stage (III-IV), lymph node metastasis, distant metastasis, preoperative CEA > 5 ng/mL, and high IMRS as significant predictors of reduced RFS (all $P < 0.05$). Variables with P < 0.10 by univariate analysis, including differentiation grade, were subsequently included in the multivariate model. Multivariate analysis identified advanced TNM stage (III-IV vs. I-II: HR = 2.15, 95% CI 1.35-3.43, $P = 0.001$) and high IMRS (vs. low: HR = 2.81, 95% CI 1.88-4.20, $P < 0.001$) as independent predictors of shorter RFS.

*Comparison of prediction efficacy*

The predictive performance of IMRS for 2-year RFS is summarized in **Table 7**. IMRS demonstrated superior predictive performance, with a C-index of 0.79 and an AUC of 0.78, outperforming both individual gene methylation markers and the conventional biomarker CEA. At the optimal cut-off value, IMRS showed a sensitivity of 74.7% and a specificity of 73.2% (Youden index 0.479), demonstrating a balanced ability to identify recurrence risk. The positive predictive value (PPV) was 55.6% and the negative predictive value (NPV) was 86.8%, indicating that patients with low IMRS had a low likelihood of recurrence within two years. Furthermore, combining IMRS with TNM staging enhanced predictive performance, with both the C-index and AUC exceeding 0.83. Sensitivity and specificity improved to 77.0% and 76.2%, respectively, and the Youden index increased to 0.532. These findings indicate that IMRS provides independent prognostic information with added value to the existing clinical staging system.

## Discussion

This retrospective study systematically evaluated the clinical significance of IMRS derived from the methylation levels of four key immune-related genes (PD-L1, CTLA-4, IRF1, FOXP3) in CRC. The core findings indicate that the IMRS, based on circulating cfDNA in peripheral blood, not only effectively distinguishes CRC patients from healthy individuals, but also independently correlates with tumor aggressiveness and unfavorable prognosis. These results suggest that IMRS may provide prognostic value beyond traditional clinicopathologic indicators. Furthermore, this study provides a new perspective for understanding immune epigenetic dysregulation in CRC and provides a preliminary basis for developing non-invasive liquid biopsy-based prognostic tools.

We observed significantly elevated plasma methylation levels of the four immune-related genes in CRC patients compared to healthy individuals, aligning with the common occurrence of aberrant methylation in tumors. For example, it has been reported that specific

**Table 4.** Association of IMRS with clinicopathologic features in CRC patients

| Feature | Low IMRS Group (n = 150) | High IMRS Group (n = 150) | Statistic | *p*-value |
|---|---|---|---|---|
| Age (years), median (IQR) | 63 (55-70) | 64 (57-71) | Z = -0.92 | 0.357 |
| Male sex, n (%) | 83 (55.3%) | 89 (59.3%) | $\chi^2$ = 0.52 | 0.471 |
| Tumor Location, n (%) | 95 (63.3%) | 90 (60.0%) | $\chi^2$ = 0.34 | 0.558 |
| Differentiation, n (%) | 43 (28.7%) | 56 (37.3%) | $\chi^2$ = 2.56 | 0.11 |
| TNM Stage (III-IV), n (%) | 66 (44.0%) | 99 (66.0%) | $\chi^2$ = 13.92 | 0.002 |
| T Stage (T3-T4), n (%) | 89 (59.3%) | 109 (72.7%) | $\chi^2$ = 5.94 | 0.015 |
| Lymph Node Metastasis (Positive), n (%) | 61 (40.7%) | 84 (56.0%) | $\chi^2$ = 7.82 | 0.005 |
| Distant Metastasis (M1), n (%) | 18 (12.0%) | 39 (26.0%) | $\chi^2$ = 10.37 | 0.001 |
| Preoperative CEA (> 5 ng/mL), n (%) | 48 (32.0%) | 70 (46.7%) | $\chi^2$ = 7.07 | 0.008 |

Note: IMRS: Integrated Methylation Risk Score; IQR: Interquartile Range; CEA: Carcinoembryonic Antigen; TNM: Tumor-Node-Metastasis. Continuous variables were compared with the Mann-Whitney U test (Z statistic), and categorical variables with the Chi-square test ($\chi^2$ statistic).

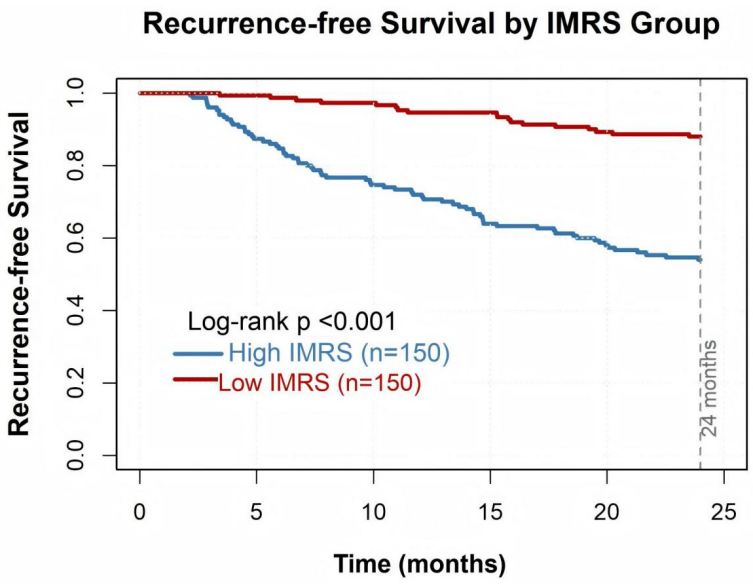


**Figure 2.** Kaplan-Meier survival curves for recurrence-free survival (RFS) stratified by high and low IMRS in patients with colorectal cancer.

CpG islands of the PD-L1 gene are hypermethylated in CRC tissues and that they are associated with poor prognosis [19]. More importantly, by integrating these individual molecular alterations into a composite IMRS, we identified significant associations with multiple clinicopathologic features of tumor progression. Elevated IMRS was significantly correlated with advanced TNM stage, deeper tumor invasion (T stage), increased lymph node and distant metastasis rates, and elevated CEA levels. These associations suggest that the IMRS reflects underlying tumor biological aggressiveness rather than representing isolated molecular alterations.

In terms of prognostic value, survival analyses demonstrated that patients with high IMRS had a significantly reduced 2-year RFS rate compared to those with low IMRS. Multivariate Cox regression analysis confirmed that high IMRS remained an independent predictor of shorter RFS (HR = 2.81). These results suggest that IMRS provide novel prognostic information beyond existing clinical staging systems.

In terms of predictive performance, the concordance index (C index) of IMRS (0.79) was higher than that of TNM stage (0.72) and CEA (0.64). Moreover, IMRS in combination with TNM stage further improved predictive accuracy (C index = 0.83), highlighting the incremental prognostic value of IMRS. This is consistent with emerging evidence in the field of liquid biopsy, where blood-based polygenic methylation markers have shown better performance than single markers for early CRC detection and prognostic prediction [20].

Our findings are consistent with previous studies on the mechanisms of immune surveillance and immune escape in tumor microenvironment. PD-L1 and CTLA-4 are classical immune checkpoint molecules, and their up-regulation represents a key strategy by which tumor cells evade T cell-mediated cytotoxicity. Studies

**Table 5.** Comparison of Recurrence-Free Survival (RFS) in CRC patients stratified by IMRS (Kaplan-Meier Analysis)

| Group | Total (n) | Events (n) | 2-Year RFS Rate (%) | Log-rank $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| Low IMRS Group | 150 | 18 | 88.0 | 36.21 | < 0.001 |
| High IMRS Group | 150 | 69 | 54.0 | | |

Note: IMRS, Integrated Methylation Risk Score; RFS, Recurrence-Free Survival. An event was defined as radiologically or pathologically confirmed recurrence, metastasis, or death from any cause. The *p*-value was calculated using the Log-rank test.

**Table 6.** Cox regression analysis of factors affecting Recurrence-Free Survival (RFS) in colorectal cancer patients

| Variable | Univariate Analysis | | Multivariate Analysis | |
|---|---|---|---|---|
| | HR (95% CI) | *p*-value | HR (95% CI) | *p*-value |
| Age (≥ 65 vs. < 65 years) | 1.22 (0.81-1.84) | 0.341 | - | - |
| Gender (Male vs. Female) | 1.08 (0.71-1.64) | 0.72 | - | - |
| TNM Stage (III-IV vs. I-II) | 3.02 (1.94-4.70) | < 0.001 | 2.15 (1.35-3.43) | 0.001 |
| Differentiation (Poor/Undiff. vs. Well/Mod.) | 1.48 (0.97-2.26) | 0.069 | 1.31 (0.85-2.02) | 0.218 |
| Lymph Node Metastasis (Positive vs. Negative) | 2.45 (1.59-3.78) | < 0.001 | 1.42 (0.85-2.38) | 0.183 |
| Distant Metastasis (M1 vs. M0) | 2.88 (1.85-4.49) | < 0.001 | 1.61 (0.93-2.78) | 0.089 |
| Preop. CEA (> 5 vs. ≤ 5 ng/mL) | 1.96 (1.30-2.96) | 0.001 | 1.38 (0.90-2.12) | 0.138 |
| IMRS (High vs. Low) | 3.40 (2.20-5.25) | < 0.001 | 2.81 (1.88-4.20) | < 0.001 |

Note: HR, Hazard Ratio; CI, Confidence Interval; IMRS, Integrated Methylation Risk Score; CEA: Carcinoembryonic Antigen; TNM: Tumor-Node-Metastasis. Univariate analysis assessed the association of each variable with RFS separately. Multivariate analysis included all variables with $P < 0.1$ from univariate analysis for adjustment. Patients were dichotomized into High/Low IMRS groups using the median score.

have shown that hypomethylation of promoter regions in these genes is usually associated with increased gene expression [21-23]. However, the paradoxical observation of elevated cfDNA methylation levels in plasma may reflect the release of DNA fragments with specific methylation patterns into the bloodstream after the massive death of relevant immune cells. These sources may include both tumor cells and immune cells within the tumor microenvironment, such as tumor-infiltrating lymphocytes and regulatory T cells. This suggests that plasma cfDNA methylation profiles likely represent a complex composite signal, differing from tissue-based methylation patterns, and may better capture systemic epigenetic alterations related to tumor burden and immune status. IRF1 is a pivotal transcription factor in Th1-type immune responses, while FOXP3 regulates regulatory T cell (Treg) development and function [24, 25]. Studies indicate that changes in FOXP3 methylation in peripheral blood or tumor-infiltrating lymphocytes are associated with modulation of Treg activity and clinical outcomes in CRC patients [26]. In addition, hypermethylation of CpG sites in the IRF1 promoter region may lead to gene silencing and impaired anti-tumor immune responses [27]. Thus, elevated IMRS may reflect a more immunosuppressive or dysregulated tumor-host interaction, providing a possible biological explanation for the observed association with poorer prognosis. Compared to previous studies, which have focused primarily on the prognostic value of single or limited gene methylation markers, the results have often been inconsistent or demonstrated limited predictive performance. For example, the prognostic value of CTLA-4 promoter methylation appears to be tumor type-dependent: CTLA-4 hypomethylation has been associated with improved response to immunotherapy in melanoma and renal cell carcinoma [28, 29], whereas its role in CRC remains unclear [30]. In contrast, the integrated scoring strategy adopted in this study may more comprehensively capture the heterogeneity and complexity of the tumor immune microenvironment by incorporating multiple functionally interrelated immune-regulatory genes. This multi-gene strategy enables more robust prognostic stratification and aligns with the current paradigm shift in precision

**Table 7.** Predictive performance of various indicators for RFS in CRC patients

| Predictive Indicator | AUC (95% CI) | C-index | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Youden Index |
|---|---|---|---|---|---|---|---|
| PD-L1 Methylation | 0.66 (0.60-0.72) | 0.67 | 62.1 | 65 | 42.5 | 80.2 | 0.271 |
| CTLA-4 Methylation | 0.62 (0.56-0.68) | 0.63 | 58.6 | 62.9 | 39.8 | 78.5 | 0.215 |
| IRF1 Methylation | 0.60 (0.54-0.66) | 0.61 | 56.3 | 60.6 | 37.9 | 76.8 | 0.169 |
| FOXP3 Methylation | 0.64 (0.58-0.70) | 0.65 | 60.9 | 64.1 | 41 | 79.6 | 0.25 |
| Preoperative CEA | 0.63 (0.57-0.69) | 0.64 | 59.8 | 63.5 | 40.5 | 79 | 0.233 |
| TNM Stage | 0.71 (0.66-0.76) | 0.72 | 66.7 | 70.4 | 49.2 | 83.1 | 0.371 |
| IMRS | 0.78 (0.73-0.83) | 0.79 | 74.7 | 73.2 | 55.6 | 86.8 | 0.479 |
| IMRS + TNM Stage | 0.82 (0.77-0.87) | 0.83 | 77 | 76.2 | 59.1 | 88.3 | 0.532 |

Note: AUC: area under the time-dependent ROC curve; PPV: positive predictive value; NPV: negative predictive value; PD-L1, programmed death-ligand 1; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IRF1, interferon regulatory factor 1; FOXP3, forkhead box protein P3; IMRS, Integrated Methylation Risk Score; CEA: Carcinoembryonic Antigen; TNM: Tumor-Node-Metastasis; Youden Index = Sensitivity + Specificity - 1.

medicine from single marker to integrative molecular signatures.

The results of this study have clear translational potential. First, the IMRS score is derived from peripheral blood and represents a typical liquid biopsy-based technique. Compared to tissue-based assays that require invasive procedures, blood-based detection offers advantages of repeatability and dynamic monitoring, thereby better capturing tumor heterogeneity and systemic disease status. For postoperative patients, IMRS may serve as a valuable complement to traditional imaging and serum tumor markers (such as CEA) for recurrence risk stratification. Within the same TNM stage, patients with elevated IMRS may be at higher risk of recurrence, suggesting the need for intensified surveillance or consideration of more aggressive adjuvant treatment strategies. Second, the immune-related gene pathways incorporated into IMRS are closely linked to current advances in tumor therapy, particularly immune checkpoint inhibitor (ICI) treatment. Although ICIs have demonstrated remarkable efficacy in CRC with deficient mismatch repair (dMMR), their therapeutic benefit remains limited for the majority of patients with proficient mismatch repair (pMMR). Currently, effective biomarkers for identifying patients most likely to benefit from ICI therapy remain lacking. Whether the immune-related epigenetic landscape reflected by IMRS is correlated with tumor responsiveness to immunotherapy represents an important and promising area for future research. It has been suggested that plasma methylation profile of specific immune-related genes may be associated with therapeutic response to ICI in malignancies such as melanoma [31], which provides a reference for further exploration in CRC.

### Limitations

This study has several limitations. First, its single-center retrospective design may have introduced selection bias. Despite multivariate adjustment, residual confounding cannot be completely excluded. Second, while the sample size (n = 300) was sufficient for internal validation, it needs robust external validation. Therefore, the generalizability and stability of the IMRS require confirmation in larger, multicenter, prospective cohorts. Third, the dichotomization of IMRS into high- and low-risk groups was based on the median value, representing an exploratory approach. The optimal clinical cutoff for IMRS requires further determination and validation using outcome-driven methods in prospective studies. Fourth, only four preselected immune-related genes were included in the present analysis. Although these genes are biologically relevant, the epigenetic regulatory network of tumor immunity in CRC is extremely complex, and additional or alternative biomarkers may further improve predictive performance. Fifth, this study primarily explored prognostic associations. The relationship between IMRS and treatment response, especially to immunotherapy, as well as the potential role of dynamic changes in IMRS during treatment, warrant further investigation.

Future research should focus on several key directions: ① validation of the optimal IMRS cutoff in large-scale, multicenter prospective cohorts; ② external validation of its prognostic performance across diverse populations; ③ expansion of the immune-related gene panel using unbiased approaches, such as genome-wide methylation profiling; ④ prospective evaluation of the association between IMRS and response to immunotherapy; ⑤ dynamic monitoring of IMRS during treatment; and ⑥ elucidation of the biological mechanisms linking IMRS to tumor progression through integrative multi-omics analyses.

## Conclusions

This study developed a novel IMRS based on circulating cfDNA methylation profiles of multiple immune-related genes. The IMRS score was associated with aggressive clinicopathologic features of CRC and independently predicted the risk of postoperative recurrence. These findings suggest that IMRS may serve as a complementary molecular biomarker with incremental prognostic value beyond conventional clinical indicators. Despite the inherent limitations of retrospective studies, our findings provide a reference for the application of liquid biopsy-based epigenetic markers in prognostic evaluation of CRC. This highlights the importance of immune-related epigenetic dysregulation in CRC progression and supports the future development of blood-based biomarkers for individualized risk stratification and treatment guidance.

## Disclosure of conflict of interest

None.

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**Supplementary Table S1.** Primer Sequences for MSP

| Gene | Forward primer (5'→3') | Reverse primer (5'→3') |
|---|---|---|
| CTLA-4 | CTCATGTACCCACCGCCATACT | TTGATGGGAATAAAATAAGGCTGAA |
| IRF1 | ATCAGCAGCCAGAGGGTAGA | CTGGCAAAAGCATCTGTGAA |
| PD-L1 | ATATAAAATAAATAATCATTCTTATACG | CGTTTAGGGATTTTGGATTTGTTTAGC |
| FOXP3 | GTATTTGGGTTTTGTTGTTATAGTTTTC | TACAAAACAAAACAACCAATTCTCG |
| ACTB | TGGTGATGGAGGAGGTTTAGTAAGT | AACCAATAAAACCTACTCCTCCCTTAA |